Puerarin accelerates peripheral nerve regeneration

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Abstract

This study investigates the effect of puerarin (PR) on peripheral nerve regeneration *in vitro* and *in vivo*. The *in vitro* study found that PR at concentrations of 1, 10, and 100 μM significantly promoted survival and outgrowth of cultured Schwann cells, as compared with controls treated with culture medium only. The *in vivo* study evaluated peripheral nerve regeneration across a 15-mm gap in the sciatic nerve of rats using a silicone rubber nerve chamber filled with PR solution. Control group chambers were filled with normal saline only. At the end of 8 weeks, animals in the PR groups, especially at a concentration of 1 μM, had a significantly higher density of myelinated axons, greater evoked action potential area, and a larger nerve conductive velocity, as compared with controls. All experimental results indicate that PR treatment promotes nerve growth and is a promising herbal medicine for recovery of regenerating peripheral nerves.

Keywords: Puerarin; Silicone rubber; Peripheral nerve regeneration

Introduction

In anastomosis, gap length is the most important factor when determining the best method for nerve repair (Zhang and Yannas, 2005). End-to-end anastomosis has

been applied to repair small gaps between severed nerve stumps; however, this method is not always appropriate, such as when bridge gaps are large (Marshall *et al.*, 1989). To deal with large gaps, nerve grafting methods were developed. An autograft is the best option for repairing injured nerves because it does not cause severe tissue reactions during the implantation period. Nevertheless, disadvantages of autografts are difficulty acquiring a donor nerve for grafting, and the inevitable risks associated with surgery at another site. Artificial nerve conduits overcome these problems (Chang *et al.*, 2009; Lu *et al.*, 2009; Yeh *et al.*, 2010). Nerve-bridging techniques introduce the proximal and distal ends of a severed nerve into a tubular structure that can guide nerve fibers along appropriate paths via mechanical orientation and confinement, and enhances stump approximation precision.

Combining traditional Chinese medicine and a nerve-bridging technique for nerve regeneration is another promising approach. For example, *Pueraria lobata* promotes regeneration of dissected rat sciatic nerve (Chen *et al.*, 2008). *Pueraria lobata* is rich in isoflavonoids, and has been used to treat angina pectoris, hypertension, influenza, neck stiffness, diarrhea, and deafness (Lin *et al.*, 2010). Puerarin (PR), the main active component extracted from dried *Pueraria lobata* roots, also has various pharmacological activities, including anti-hypertensive, hypolipidemic, anti-diabetic, cardioprotective, and estrogen-like effects, and promotes

recovery from alcohol abuse (Li *et al.*, 2010; Teng *et al.*, 2009; Zhou *et al.*, 2010). Although literature indicates that PR has various functions, to the best of our knowledge its effect in promoting neuro-growth has never been characterized. Therefore, this study uses the Schwann cell line, which has been widely adopted in studies of cell differentiation and neurite outgrowth (Cornejo *et al.*, 2010), to investigate its neuronal characteristics after exposure to PR solution. Additionally, as the regenerative capacity of the peripheral system in mammals is so efficient for short gaps that the benefits of PR may not be fully revealed. Therefore, this study used a silicone rubber chamber filled with PR solution to establish a nerve bridge across a 15-mm gap in rat sciatic nerves. Histological and electrophysiological techniques were applied to evaluate nerve function recovery.

Materials and Methods

Cell culture and treatment

Schwann cells (RSC96) were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in Dulbecco's modified media (DMEM) medium supplemented with 5% fetal calf serum, 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 2 mM L-glutamine. The Schwann cells (7×10^3 cells/cm^2) were then induced to undergo neuronal differentiation via treatment with 1, 10, and 100 μM PR

solution for 2 days. The PR was obtained from Sigma-Aldrich, Inc. (#P5555; St. Louis, MO, USA) and its purity was about 80%. Cells exposed to the vehicle alone (culture medium only) comprised the control group. Six replicates were used in each study.

Analysis of cell viability

After cell incubation for 24 h, the medium was removed, replaced with 110 μL/well of 5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution in 1×phosphate buffered saline (PBS) and further incubated in an incubator at 37°C for 4 h. The MTT solution was then removed and replaced with 50 μL dimethyl sulfoxide (DMSO) to dissolve the formazan. Color intensity was determined using a microplate reader (ELx800TM; Bio-Tek Instrument, Inc., Winoski, VT, USA) at 550 nm absorbance. Data are expressed as a percentage of the control level of optical density within individual experiments.

Cell imaging

After treatment with the PR solutions for 48 h, Schwann cells were washed with PBS twice, fixed in 2% paraformaldehyde for 30 min, and then permeabilized with 0.1% Triton X-100/PBS for 30 min at room temperature. After washing with PBS, the

terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was applied according to the manufacturer's instructions (Boehringer Mannheim, city, country). Cells were incubated in TUNEL reaction buffer in a 37°C humidified chamber for 1 h in darkness, and then rinsed twice with PBS and incubated with 1 mg/ml 4',6-diamidino-2-phenylindole (DAPI) at 37°C for 10 min. Stained cells were visualized using a fluorescence microscope (Olympus DP70/U-RFLT50; Olympus Optical Co., Ltd., Japan). The DAPI- and TUNEL-positive cells were counted as live and apoptotic cells, respectively.

Surgical preparation of rats

Forty adult Sprague-Dawley rats were implanted with silicone chambers. Rats were anesthetized via inhalation of an anesthetic (AErrane®, Baxter, USA). Following skin incision, fascia and muscle groups were separated by blunt dissection, and the right sciatic nerve was severed into proximal and distal segments. The proximal stump was then secured with a single 9-0 nylon suture through the epineurium and outer wall of the silicone rubber chamber (1.47 mm ID, 1.96 mm OD; Helix Medical, Inc., Carpinteria, CA, USA). Rats were divided into four groups. Chambers in group A (n = 10), the control group, were filled with normal saline. In group B (n = 10), chambers were filled with 1 μM PR solution. Similarly, chambers in groups C (n = 10)

and D (n = 10) were filled with 10 and 100 μM PR solution, respectively.

Chamber lumen volume was approximately 25.5 μl. These liquid fillings were micropipetted into the lumens by passing the tip of the needle into the silicone rubber chambers. The distal stump was then secured into the other end of the chamber. Both proximal and distal stumps were secured to a depth of 1 mm into the chamber, leaving a 15-mm gap between stumps. The muscle layer was re-approximated with 4-0 chromic gut sutures, and skin was closed with 2-0 silk sutures. All rats were then returned to plastic cages with a sufficient amount of sawdust to eliminate direct contact between the paralyzed limb and cage floor. These rats were housed in controlled rooms at 22°C with 45% humidity and 12-h light cycles. Rats had access to food and water *ad libitum*. All animals were maintained in facilities approved by the China Medical University for Accreditation of Laboratory Animal Care and in accordance with current National Science Council regulations and standards.

Electrophysiological techniques and statistical analysis

After regeneration for 8 weeks, animals were re-anaesthetized and their sciatic nerves exposed. The stimulating cathode was a stainless-steel monopolar needle placed directly on the sciatic nerve trunk, 5-mm proximal to the transection site. The anode was another stainless-steel monopolar needle placed 3-mm proximal to the

cathode. Amplitude, area, and nerve conductive velocity (NCV) of evoked muscle action potentials (MAPs) were recorded from gastrocnemius muscles by micro-needle electrodes linked to a computer (Biopac Systems, Inc., USA). The amplitude and area under the MAP curve were calculated from baseline to maximal negative peak. The NCV was determined by placing recording electrodes in gastrocnemius muscles and stimulating the sciatic nerve proximally and distally to the silicone rubber conduit. The NCV was then calculated by dividing the distance between stimulating sites by the difference in latency times. All data are expressed as mean ± standard deviation. Statistical comparisons between groups used one-way analysis of variance.

Histological techniques and statistical analysis

Sciatic nerve sections were extracted from the middle of regenerated nerves in chambers. Following fixation, nerve tissue was post-fixed in 0.5% osmium tetroxide, dehydrated, and embedded in spurs. The tissue was then cut to a thickness of 5 μm using a microtome with a dry glass knife, and stained with toluidine blue. All tissue samples were examined under a light microscope (Olympus IX70; Olympus Optical Co., Ltd., Japan). An image analyzer system (Image-Pro Lite; Media Cybernetics, USA), coupled to the microscope counted the blood vessels and calculated the cross-sectional area of each nerve section at magnifications of 40–400×. At least

30–50% of the nerve section area was randomly selected from each nerve specimen and its axons were counted under 400× magnification. Axon counts were extrapolated using the area algorithm to approximate total number of axons in each nerve. All data are expressed as mean ± standard deviation. Statistical comparisons between groups used one-way analysis of variance with the Scheffe test.

Results

Spindle-shaped cellular morphology of Schwann cells cultured on a culture plate was viable with no infection signs. Treatment with PR solution did not induce apoptotic cell death, as nearly no TUNEL-positive cells existed, suggesting that DNA fragmentation did not occur in these Schwann cells (Fig. 1a). Conversely, the optical density of Schwann cells treated with the PR solution in groups B, C, and D was significantly increased compared with that of controls in group A (Fig. 1b), indicating that the PR solution exerted growth-promoting effects on cultured Schwann cells.

Figures 2a–2d show representative cross sections of regenerated nerve specimens. Regenerated nerves selected from all tube-grafted groups had similar ultrastructural organizations. The epineurial and perineurial regions of regenerated nerves consisted mainly of a collagenous connective tissue matrix with circumferential cells resembling perineurial cells and fibroblasts. Additionally, nerve fibers and blood

vessels were loosely distributed in the endoneurium. Nuclei of Schwann cells were interspersed among these nerve fibers. Axons in the endoneurium were easily defined by their surrounding myelin sheaths stained dark blue by toluidine blue. Morphometric data reveal that 1 and 10 μM PR solution significantly increased the density of myelinated axons that grew into the distal end of the chamber by approximately two-fold, as compared with controls (Fig. 3).

Figures 4a–4b show light microscopic images of the gastrocnemius muscle. Compared with normal muscle fibers, those from injured sites of rats in all groups were smaller and rounder and had a looser structure with a dramatically increased number of nuclei, indicating that muscle atrophy was still considerable even after recovery for 8 weeks.

Electrophysiological tests performed during follow-up after surgery indicate successful reinnervation of the gastrocnemius muscle by the regenerating sciatic nerve with obvious excitability and conductivity in all rats in all four groups (Fig. 5). Compared with controls (group A), the PR-treated animals in groups B (1 μM), C (10 μM), and D (100 μM) had a relatively larger MAP amplitude and area. Specifically, the difference in MAP area between group A and group B was significant at $P<0.05$ (Fig. 6). Additionally, group B animals had a significantly higher NCV than that of groups C, D and A.

Discussion

Most studies suggested that the critical nerve defect in rats is 10 mm (Liu *et al.*, 2011; Yang *et al.*, 2011). In our opinion, a nerve gap exceeding 10 mm should be utilized to fully reveal the benefits of modifications in the bridging chamber. This study therefore filled silicone rubber chambers with PR solution to repair a 15-mm gap in dissected rat sciatic nerves. Notably, PR is the predominant isoflavone glycoside in *Pueraria lobata* (wild) Ohwi (Rong *et al.*, 1998), which has been used to treat acute ischaemic stroke, heart disease, and systemic disorders (Bo *et al.*, 2005). Experimental studies with animals have shown that PR increases cerebral blood flow, attenuates cerebral injury resulting from ischemia and reperfusion, and improves learning and memory (Sang *et al.*, 2004; Xu *et al.*, 2004; Xu *et al.*, 2007). These studies showed that PR may have positive effects on the nervous system. In this study, administration of an appropriate PR dosage exerted growth-promoting effects on cultured Schwann cells, and significantly enhanced the maturity of regenerated nerves in silicone rubber chambers. Notably, groups with PR dosages of 1 and 10 μM had significantly higher densities of axons that successfully grew across the 15-mm gap than controls. Several mechanisms related to the growth-promoting effects of PR have been proposed. The neuroprotection capability of PR may be through its anti-oxidant

(Zhang *et al.*, 2008), anti-inflammatory (Kim *et al.*, 2010), and anti-apoptic properties (Xu *et al.*, 2005). These neuroprotection effects by PR can simultaneously attenuate injured nerve degeneration and boost regeneration. Another possible explanation is that PR decreases the Ca^{2+} concentration in neurons, preventing neurochemical damage (Xu and Zheng, 2007).

While the capacity of PR to potentiate axonal outgrowth in developing nerves is uncontested, PR needs to be utilized carefully in clinical applications. This study shows that the growth-promoting capability of PR was suppressed when its concentration was 100 μM. We believe that an excessive PR dosage induced adverse effects on regenerated nerve recovery. This finding is similar to that reported by Boyd and Gordon (2002), indicating that axonal regeneration can be inhibited by administering high doses of brain-derived neurotrophic factors by functional blockage of $p75^{NTR}$ receptors. Mohiuddin *et al*. and Hirata *et al*. also demonstrated that excessive nerve growth-promoting substances can suppress axotomy-induced elevation of growth-associated protein 43 (GAP-43), resulting in inappropriate reestablishment of injured nerves (Hirata *et al.*, 2002; Mohiuddin *et al.*, 1999).

This motor nerve conduction study found that applying PR, especially 1 μM PR, significantly increased the MAP area and NCV, as compared with those of controls. Since MAPs are believed to reappear when regenerating myelinated nerve fibers

reach their target organ (Werdin *et al.*, 2009), accelerated NCV may be attributed to increased axonal growth rate in PR-treated animals. This study identified large variations in MAP curve amplitude, likely resulting from considerable gastrocnemius muscle atrophy, even though muscle fibers were reinnervated during recovery for 8 weeks.

In conclusion, experimental findings suggest that PR solution increased cultured Schwann cell viability and accelerated axonal re-growth and functional recovery in rats. Experimental data provide insight into the effect on neuro-regeneration of this ancient drug. Further studies of PR neuro-regeneration mechanisms are needed for definite conclusions.

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Captions

Figure 1: (a) Nuclei of Schwann cells characterized by DAPI and TUNEL assays and investigated via fluorescent microscopy. (b) Quantification of viability of Schwann cells treated with PR solution relative to controls. Values are mean±standard error.

Figure 2: Light micrographs of regenerated nerve cross sections from group A (panel a, saline), group B (panel b, 1 μM PR), group C (panel c, 10 μM PR), and group D (panel d, 100 μM PR). Numerous myelinated axons (arrows) and blood vessels (arrowheads) are dispersed in the endoneurium. Scale bars = 30 μm.

Figure 3: Morphometric analysis of regenerated nerves, including (a) axon numbers, (b) axon area, (c) axon density, and (d) total nerve area. Values are mean±standard error. *$P<0.05$ indicates a significant difference from other

groups.

Figure 4: (a) Normal rat gastrocnemius muscle. The muscle bundle cross section has closely packed fibers. Nuclei exist at the periphery. (b) A typical muscle section seen in the groups with or without PR treatment. Evident muscle atrophy is noted with increased fibrosis and fatty infiltration. Scale bars = 20 μm.

Figure 5: Representative recordings of evoked MAPs in gastrocnemius muscles from groups A–D. Obvious excitability and conductivity exists for all rats in all four groups.

Figure 6: Analysis of evoked MAPs, including (a) peak amplitude, (b) area under the MAP curves, and (c) NCV. Values are mean±standard error. *$P<0.05$ indicates significant difference from other groups.